



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 5 :</b> A61K 31/16, 31/155 C07C 237/06, 257/14	<b>A1</b>	<b>(11) International Publication Number:</b> WO 93/12777 <b>(43) International Publication Date:</b> 8 July 1993 (08.07.93)
<b>(21) International Application Number:</b> PCT/US92/11352 <b>(22) International Filing Date:</b> 31 December 1992 (31.12.92)  <b>(30) Priority data:</b> 817,900                      3 January 1992 (03.01.92)                      US  <b>(71) Applicant:</b> NEW YORK UNIVERSITY [US/US]; 550 First Avenue, New York, NJ 10016 (US).  <b>(72) Inventors:</b> CHERKSEY, Bruce, D. ; 608 Garden Street, Hoboken, NJ 07073 (US). LLINAS, Rodolfo, R. ; 16 Sutton Place, New York, NY 10021 (US). SUGIMORI, Mutsuyuki ; 30 Waterside Plaza, New York, NY 10010 (US).  <b>(74) Agents:</b> ROBINSON, Joseph, R. et al.; Darby & Darby P.C., 805 Third Avenue, New York, NY 10022 (US).		<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> USE OF POLYAMINES AS IONIC-CHANNEL REGULATING AGENTS  <b>(57) Abstract</b>  Methods for regulating cation transport across cellular membranes possessing cation channels are provided. The cell membrane possessing a specific ion channel is exposed to a non-aromatic polyamine compound having a lysine- or arginine-based moiety (or a guanidine moiety) coupled to a straight chain polyamine. These polyamines as well as a method for regulating NMDA through a spermidine-like effect on NMDA receptors by exposing a cell membrane to selected polyamines are also provided.		

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10        USE OF POLYAMINES AS IONIC-CHANNEL REGULATING AGENTS

          This application is a continuation in part of (a) of U.S. Patent Application Serial No. 219,105 filed July 14, 1988, in turn a continuation-in-part of U.S. Patent Application Serial No. 154,845 filed February 10, 1988 (now abandoned); (b) 15 PCT Application PCT/US89/00558 (published under WO89/07608) filed on February 10, 1989 (now U.S. Application Serial No. 435,488 filed October 10, 1989), now abandoned, (c) U.S. Application Serial No. 375,776, filed July 3, 1989, now abandoned, and (d) PCT/US90/03771, filed on July 3, 1990. The 20 United States Government has rights in this invention by virtue of Grant Nos. NS-13742 from the National Institute of Neurological and Communicative Disorders and Stroke, NIH EY-08002 from the National Eye Institute, U.S. Public Service, National Institutes of Health, AGE-A1P01AG09480 from the 25 National Institute on Aging, and AFOSR 89-0270 from the U.S. Air Force, Department of Defense.

FIELD OF THE INVENTION

          This invention relates generally to the use of polyamine compounds as agents regulating ionic conductances in 30 cellular membranes. These compounds have sperr-dine-like effects on NMDA receptors and, as such, may act as NMDA regulators. Certain of these compounds also have separate and specific ion channel effects. These effects, in general, and particularly the ion channel effects, are believed to be of 35 therapeutic value.

BACKGROUND OF THE INVENTION

          Transport of charged particles across cell membranes

is mediated (and in substantial part, regulated) by membrane proteins (including those referred to as "voltage-sensitive" or "voltage-dependent" channels as well as "ligand-gated" channels).

5           In this discussion the terms "channel" and "channel protein" are used interchangeably without implying that a channel must necessarily consist of a single protein, although channels that have been isolated are believed to be single proteins.

10           Most channel proteins mediate the transport of one ionic species with substantially higher specificity than transport of other ionic species. It is common to name various channels after the ion for which they are specific: thus, there are sodium channels, potassium channels, calcium channels, etc.

15           In turn, ionic channels specific for the transport of one cation, may be further divided into various subcategories or channel types, based on the way they interact in response to electrical and/or chemical (pharmacological) stimuli. Calcium channels in particular, which have been identified in a number  
20 of different cell types, including neurons, appear to have differences (as well as similarities) in morphology, properties and/or function. Other such differences have not been directly related to cell types; in fact, different types of channels are normally present in the same cell.

25           Historically, neuronal calcium conductances were first divided into two categories based on the driving force that activates them: the high-threshold calcium conductance (HTCC) and the low-threshold calcium conductance (LTCC). In central neurons, HTCC is more prominent in the dendrites and  
30 LTCC is more prominent in the soma or cell body. Later, neuronal calcium channels were grouped into three categories: the T-channels, which are believed to be responsible for LTCC; the N-channels, the conductance properties of which showed imperfect correspondence to HTCC; and the L-channels which are  
35 not commonly represented in central neurons but of which the

conductance properties also showed correspondence to HTCC.

These three categories can be further distinguished by differences in pharmacological properties. The L-channels are dihydropyridine-sensitive, i.e. they are effectively  
5 blocked by dihydropyridines, such as nifedipine and nitrendipene, whereas the T- and N-channels are dihydropyridine-resistant. To a large extent, some T-channels resist blockage by cadmium ions; more importantly, some T-channels are specifically blocked by alcohols (especially octanol) at  $10^{-4}$  M  
10 or lower concentrations. Finally, the N-channels are said to be blocked by omega-conotoxin, a toxin isolated from the venom of the marine snail Conus geographicus, to which the L- and T-channels are resistant. Miller, R.J. Science, 1987, 235: 46-52.

15 However, the calcium channels responsible for the HTCC (both the calcium-dependent plateau potential component and the dendritic spike component of the HTCC) in Purkinje cells are activated at -50 mV, and are dihydropyridine-insensitive and also omega-conotoxin-resistant. These channels are  
20 specifically blocked by a low-molecular weight blocking agent isolated from the venom of funnel-web spiders. Llinas et al., Proc. Nat'l Acad. Sci. USA, 1989, 86:1689-1695. Furthermore, the dihydropyridine- and conotoxin-resistant calcium channels appear to be absent from inferior olivary and thalamic neurons.  
25 These calcium channels have been called P-channels because they were first described in Purkinje cells. Llinas, et al., Ann. N.Y. Acad. Sci., 1989, 560:103-111. P-type channels have also been shown to exist in squid giant synapse and squid optic lobe.

30 Sodium and potassium channels are also of various types. See generally Hille, B. "Ionic Channels of Excitable Membranes," Sinauer Assoc. Inc., Sunderland, Mass., 1984.

Two main types of sodium channels are found in electrically excitable cells such as central neurons. One type  
35 of channel, responsible for the so-called "fast" sodium conduc-

tance, is specifically blocked by tetrodotoxin (TTX), a toxin isolated from puffer fish. A second type of sodium channel, responsible for the slow sodium current, is blocked by local anesthetic agents such as lidocaine. A distinctly different sodium channel is located in non-electrically excitable tissue such as epithelium, and is not blocked by TTX or the local anesthetics but is blocked by the diuretic amiloride.

The known blockers of K<sup>+</sup> channels include tetraethylammonium (TEA), aminopyridines and quinine.

Agents that block calcium channels with high affinity and/or specificity as well as agents that activate calcium channels are useful as reagents in electrophysiological research. Availability of such agents is essential for understanding calcium channel properties and function. Such agents are especially useful in the design of prototype drugs and in drug screening.

The prior art calcium-channel blocking agents do not satisfy this need for one or more of the following reasons:

(a) Lack of Affinity for Particular Calcium Channels. The P-channel in particular is resistant to: omega-conotoxin; dihydropyridine and its derivatives; and alcohols.

(b) Lack of Specificity. When developing substances that regulate a certain channel, specificity is a primary concern.

(c) Unavailability in Large Amounts at Low Cost. It would be desirable to identify channel regulating agents which could be synthesized conveniently and at low cost.

(d) Lack of Knowledge of Blocking Agent Mode of Action. The availability of blocking agents of different structure would help to elucidate the mechanism and to identify the site of blocking activity. This would in turn lead to design of improved blocking agents or of novel agents that had different calcium channel modulating properties, such as activation of calcium channels.

The calcium channel modulators of the present

invention also have potential uses as prototypic drugs exhibiting anticonvulsant (e.g. anti-epileptic), anxiolytic, tranquilizing, anti-Alzheimer's, and/or memory-improving properties.

5 Analogous needs exist for substances that alter the ion-transport properties of other ionic channels, such as sodium or potassium channels. Such substances also find therapeutic uses such as:

(1) Alteration of the function of Na<sup>+</sup> channels may  
10 be of therapeutic utility for the treatment of muscle spasms, torticollis, tremor, learning disorders, and Alzheimer's disease. Polyamines which block slow sodium channels would have additional utility as local anesthetic agents. Agents  
15 which act on the epithelial Na channel would be useful adjuncts in the treatment of cystic fibrosis and asthma and as antihypertensive agents.

(2) Drugs which modulate the activity of K<sup>+</sup> channels would be useful as protective agents against the damaging effects of anoxic and ischemic disorders and hypertension, and  
20 would act to protect red blood cells against damage in malaria and sickle-cell disease.

Calcium blockers (as well as other types of calcium modulators), used separately, are expected to yield information about how the event of cell death is organized.

25 Chideckel, E.W., et al., Br. J. Pharmacol., 1988, 89:27-33 report that transient exposure to "polyamines" causes a relaxation of guinea-pig respiratory tract smooth muscle (trachealis muscle) in response to subsequent exposure to potassium ion extracellularly. This is contrary to the normal  
30 contractile response of this muscle exhibited in the absence of "polyamines" (or other calcium-ion entry blocking drugs such as nifedipine, verapamil, diltiazem or calcium ion-free solutions). The authors hypothesize inter alia that spermidine may have a Ca<sup>2+</sup> antagonist function and that spermidine and certain  
35 other polyamines may have a calcium channel blocking activity,

"perhaps" similar to that of the known  $\text{Ca}^{2+}$ -entry blocking drugs referred to above.

Spermidine was the only polyamine tried in the experiments described in this article, although effects of  
5 other polyamines related to membrane  $\text{Ca}^{2+}$  fluxes and cellular  $\text{Ca}^{2+}$  handling are cited from other literature as follows:

- 10 \* putrescine is said to cause an increase in cytosolic  $\text{Ca}^{2+}$  concentration in heart and kidney slices attributed to both an increase in extracellular  $\text{Ca}^{2+}$  influx and a simultaneous release of  $\text{Ca}^{2+}$  from mitochondria;
- 15 \* spermine is said to inhibit spontaneous contraction of the uterus muscle in a manner than can be counteracted by increasing extracellular  $\text{Ca}^{2+}$  concentration;
- \* spermine and spermidine are said to have a relaxant effect on smooth muscles of the gut, uterus, respiratory tract and vasculature.

The authors do not mention the type of calcium  
20 channels that are said to be present in trachealis muscle (or for that matter in the other tissues mentioned.) As to trachealis muscle, dihydropyridine sensitivity indicates that L-type channels might be implicated. As to the other tissues mentioned, no claim is made in this article that calcium  
25 channels were involved (many other factors or processes could influence the concentration of calcium in the cytosol). In fact, some of the results reported in this article are inconsistent with blockage of calcium channels, and (based on the disclosure of this article) cannot be attributed to activation  
30 of calcium channels because of the many other factors that may be at work, including actions on potassium channels or direct action on smooth muscle (papaverine-like effects).

Hirsh, S.R., et al, Psychopharmacology, 1987, 83:101-  
104 report that two polyamines, spermine and spermidine,  
35 administered intraperitoneally, at doses of 5-40 mg/kg, in rats



caused a dose-dependent inhibition of spontaneous climbing and wheel running behavior, which the authors tentatively attributed to modulation by these polyamines of limbic dopamine function. Hirsch et al. report further that the mice remained alert during the period of reduced locomotor activity and appeared to be in good health.

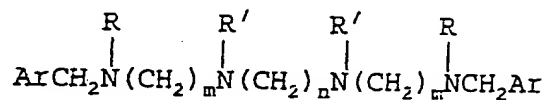
Injected intracerebrally (in the striatum), these compounds failed to induce asymmetric behavior. On the other hand, these compounds did antagonize hyperactivity (when injected into the nucleus accumbens) which the authors (erroneously according to the present inventors) considered consistent with modulation of dopamine function but not dopamine receptor blockage. No direct or indirect effect on ionic (or specifically calcium) conductance is disclosed or suggested.

The effects reported but erroneously attributed to dopamine by Hirsh et al. are hereafter referred to as "spermidine-like" effects.

Palade, P., J. Biol. Chem., 1987, 262:6149-6154 reports that the release of  $\text{Ca}^{2+}$  (pre-loaded into isolated subfractions of sarcoplasmic reticulum) usually observed upon addition of various release-inducing substances (including caffeine and thymol) could be blocked by ruthenium red, certain organic polyamines (spermine, spermidine and triethylene tetramine) certain antibiotics (neomycin, kanamycin, tobramycin, gentamicin, streptomycin and clindamycin) and certain polypeptides (polylysine, polyarginine, some histones and protamine). The authors observed that these agents have only one feature in common: the presence of several amino groups. Based on the inability of the polyamines to affect calcium pump function, the authors concluded that the effect of ruthenium red, spermine, neomycin and polylysine (which have quite diverse structures) was not due to interference with the  $\text{Ca}^{2+}$  pump but to blockage of the sarcoplasmic reticulum calcium channels. Many of these agents appeared to block calcium

release at (estimated) nanomolar concentrations. The authors also remarked that each compound's potency in inhibiting calcium release appeared to be related to the number of amine groups present in that compound: antibiotics appeared to be more potent on a molar concentration basis. However, since the experiments reported in this article measured only blockage of calcium release, they did not establish calcium channel involvement. Also, the reported results are limited to  $\text{Ca}^{2+}$  channels in the sarcoplasmic reticulum which have a large conductance (perhaps an order of magnitude greater than other known calcium channels) and, as stated by the authors, may be further limited to one type of  $\text{Ca}^{2+}$  channel in the sarcoplasmic reticulum. (The authors explicitly state that these compounds appear to be active on SR calcium channels that are insensitive to inositol triphosphate.) In general, SR calcium channels may be considered to be in a separate category not only because of their large conductance but also because of their location in the cell.

In a series of papers, Melchiorre, C., et al., report that certain polymethylene tetramines of the formula



(wherein Ar is an aromatic group; R, R' are hydrogen or methyl and m,n are various integer combinations within the range 5-14) have M-2 muscarinic receptor blocking activity in guinea-pig heart atria and intestinal ileum. Several of these compounds are said to selectively block the atrial muscarinic receptor with considerably higher affinity than the ileal receptor, and thus could possibly serve to distinguish between the two receptors. Nothing is disclosed about calcium channels, but a general synthetic scheme for the non-aromatic moieties of the disclosed polyamines is provided: J. Med. Chem., 1989, 32:79-

84; J. Med. Chem., 1985, 28:1643-1647; and Can. J. Physiol. Pharm. 21., 1980, 58:1477-1483.

International Patent Application WO89/07098 discloses butyryl-tyrosinyl spermine and various analogs thereof which are said to be (potentially) useful for treating neurodisorders associated with an etiological agent binding to a glutamate receptor.

#### OBJECTS OF THE INVENTION

The present invention has the following objects:

- 10 - to devise novel agents and methods for regulating (e.g. blocking, modulating or activating) ionic channels (including calcium, sodium, and potassium channels);
- to devise novel agents and methods for regulating transmitter release or synaptic transmission;
- 15 - to devise additional agents and methods for blocking ionic channels that overcome one or more of the disadvantages associated with known blocking agents described above;
- to use each of such regulating agents and methods to design prototypical drugs that regulate calcium, sodium, and/or
- 20 potassium channels (with emphasis on drugs that block channels of central neurons); and to increase the understanding of ionic channel structure, properties and function;
- to design more active, less costly and/or otherwise improved substitutes for such agents and/or drugs;
- 25 - to design agents that have calcium, sodium, and/or potassium channel modulating functions other than activation or blockage. For example, agents which act on the G-protein in cell membranes alter the activity of ionic channels indirectly and so do agents that act on the NMDA receptor.

30 Other objects of this invention will be apparent to those skilled in the art in light of the present specification, claims and drawings.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a tracing of spontaneous firing activity  
35 observed in the guinea-pig Purkinje cell pursuant to applica-

tion of a direct square current pulse (0.3 nA, 62 msec) in the absence of any blocking agents (control).

Figure 1B is a tracing of the response of the Purkinje cell to a 0.45 nA direct square current pulse after addition of a mixture of compounds B and R: the calcium conductance is blocked.

Figure 1C upper trace is a tracing of the response of a Purkinje cell to a 0.45 nA direct square current pulse after (i) the P-channel has been blocked with a mixture of compounds B and R, (ii) the potassium conductance has been blocked with TEA, (iii) the sodium conductance has been blocked with TTX. The remaining spike is due to a calcium channel of the dihydropyridine-sensitive type. This is demonstrated in Fig. 1C lower trace where the L-channel also is blocked by dihydropyridine or Fig. 1D (lower trace) where the L-channel is blocked by streptomycin. (The upper trace in 1D is of the same type as in 1C.)

Figure 2 is a graph of the presynaptic calcium current in the squid synapse observed after addition of various amounts of A. aperta venom in a voltage-clamp experiment demonstrating dose-dependence of this effect.

Figure 3 is a graph showing the fraction of the excitatory postsynaptic potential (EPSP) remaining (with the control taken as 1) after addition of various amounts of partially purified P-channel blocking factor from A. aperta venom in squid synapse. The results demonstrate the dose-dependence of the effect by the factor on the EPSP.

Figure 4 shows the postsynaptic action potential in squid synapse pursuant to direct stimulation of the presynaptic terminal. Tracing A was recorded prior to the addition of a mixture of compounds B and R in the bath (0.15 ml of the preparation of Example 2); tracing B was recorded 4 minutes after the addition of a mixture of compounds B and R; and tracing C was recorded 6 minutes after addition of a mixture of compounds B and R.

Figure 5 upper trace is the postsynaptic potential (EPSP) response in squid synapse in a voltage clamp experiment before (A) and after application of a mixture of compounds B and R in the bath (which already contained TTX and 3-aminopyridine). The middle trace is the presynaptic calcium current before (A) and after application of a mixture of compounds B and R in the bath. The lower trace is the applied voltage step (28 mV, 5.5 msec).

Figure 6 is the trace of the postsynaptic potential again in squid synapse after application of a 38.2 mv/10 msec voltage step in a voltage clamp experiment in the presence of TTX and 3-aminopyridine. Recordings were made 2, 6, 10 and 13 minutes after the addition of a mixture of compounds B and R.

Figure 7A and B are traces of the presynaptic and postsynaptic potential in squid synapse in the absence (upper trace) or presence (lower trace) of calcium blocker.

Figure 8 is a superimposition of traces of perineural presynaptic currents in nerve muscle preparations before (a) and after (b) the addition of 300  $\mu$ M of compound S. In (c) calcium concentration was raised in the presence of compound S.

Figure 9A traces the potassium channel activity in purified axon membrane to which a 50mV/1 msec voltage is applied, prior to the addition of any compounds according to the present invention. The traces illustrate normal activity comprised of a number of potassium channels.

Figure 9B is a single trace in two segments of the potassium channel activity in purified axon membrane to which 50 mV/1 msec voltage is applied one minute after the addition of compound (ZZ) at mM (high) concentration.

Figures 9C and 9D are traces of the potassium channel activity in purified axon membrane to which 50mV/1 msec and 75 mV/1 msec voltage, respectively, are applied three minutes after the addition of compound (ZZ) at mM (high) concentration.

Figure 10 is a trace of the sodium channel activity in purified axon membrane to which a 50 mV/1 msec voltage is

applied, prior to the addition of any compound according to the present invention.

#### SUMMARY OF THE INVENTION

One aspect of this invention is directed to a method  
5 for regulating cation transport across cellular membranes  
possessing cation channels comprising exposing a cell membrane  
possessing an ion channel of a specific type, to a nonaromatic  
polyamine compound having a lysine- or arginine-based moiety  
(or a guanidine moiety) coupled to a straight chain polyamine.

10 Another aspect of this invention is directed to a  
method for regulating NMDA through a spermidine-like effect on  
NMDA receptors comprising exposing a cell membrane to compounds  
selected among the foregoing polyamine compounds.

In addition, the present invention is directed to the  
15 foregoing polyamine compounds themselves.

Other aspects of this invention are directed to  
methods for blocking calcium channels and to methods for  
blocking synaptic transmitter release using one or more of the  
compounds referred to above.

20 Another aspect of this invention involves methods for  
regulating ionic channels, either voltage- or ligand-gated such  
as the glutamate-activated channel (NMDA), by selecting a  
compound according to the present invention that is a calcium-,  
sodium-, or potassium blocker or modulator, and exposing a cell  
25 to the presence of this compound to bring about the desired  
specific regulating result on the target channel.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The disclosure of any and all cited documents  
including literature and patent applications is incorporated by  
30 reference in its entirety.

It is understood that in the present specification  
"ionic channel regulating agents" shall mean compounds and  
compositions which regulate flow of a cation by acting directly  
on a channel or by acting indirectly on it (e.g., by acting on  
35 another substance or cellular structure which in turn influen-

ces the function of an ionic channel).

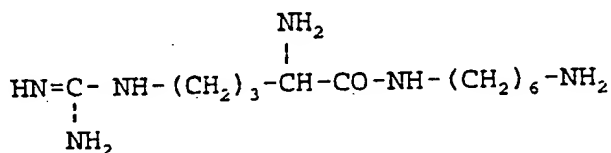
Further, "block" shall mean the interruption of ion transport, with direct or primary block being directly attributed to the effect of the blocking agent, while indirect (or secondary or dependent) block is a result of, or due to the action of, the direct block. For example, direct block of a calcium channel may effectively block a potassium channel where potassium transport is dependent upon calcium transport.

In accordance with the present invention, certain nonaromatic polyamines have the ability to block calcium channels including P-type calcium channels (i.e. channels that display one or more of the characteristics associated with the P-channels first identified in Purkinje cells and described above).

As used herein, "polyamine" will generally refer to a compound that has (a) a dehydroxylated (or decarboxylated) arginine or lysine moiety (or a guanidine moiety) appended to (b) a straight polyamine chain moiety having two or more nitrogens separated by methylene groups.

Illustrative of polyamines useful as ionic channel regulating agents are the following compounds:

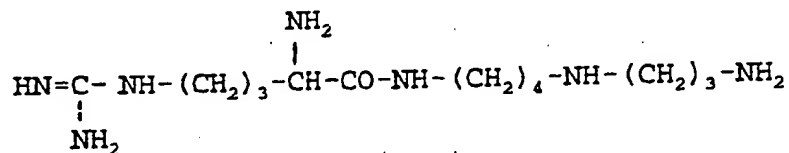
(A)



(weak potassium channel blocker -  
no activity on P-channel)

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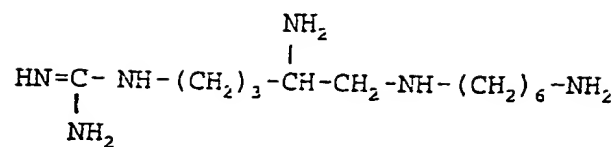
(B)



(Ca<sup>2+</sup>-channel blocker)

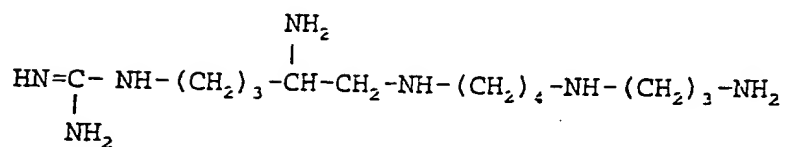
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(D)

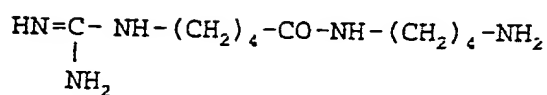


(potassium-channel blocker)

5 (E)

(Ca<sup>2+</sup>-channel blocker)

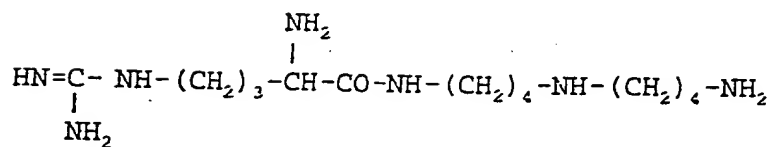
(P)



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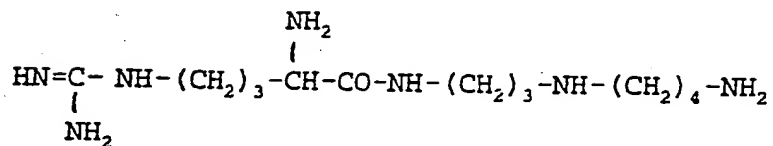
(sodium-channel blocker)

(Q)

(Ca<sup>2+</sup>-channel blocker)

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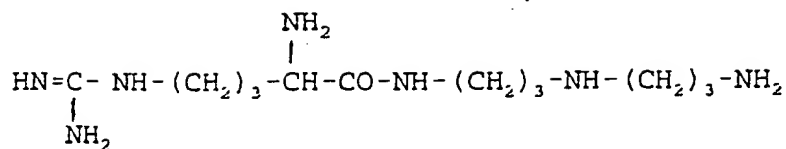
(R)

(Ca<sup>2+</sup>-channel blocker)

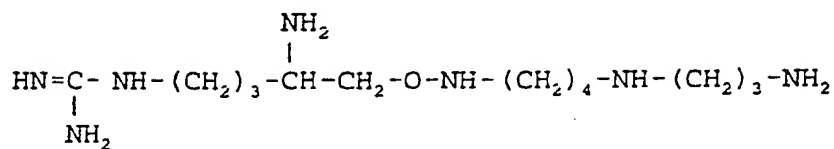
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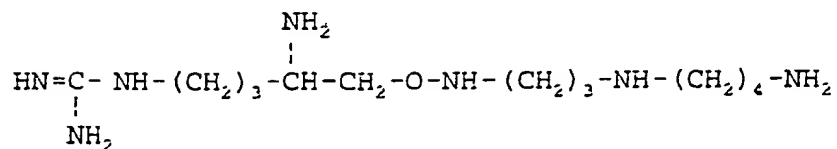
(S)

(Ca<sup>2+</sup>-channel blocker)

5 (T)

(Ca<sup>2+</sup>-channel blocker)

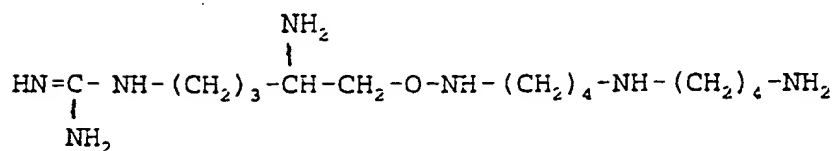
(U)



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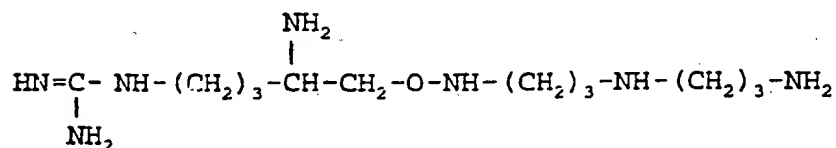
(Ca<sup>2+</sup>-channel blocker)

(V)

(Ca<sup>2+</sup>-channel blocker)

15

(W)

(Ca<sup>2+</sup>-channel blocker - spider  
venom factor (FTX)-like)

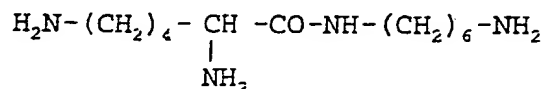
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Example of compounds having different channel

- blocking activity or displaying the same type of effect as spermidine when tested i.p. in animals (but with substantially greater potency) include derivatives or analogues of the above-listed compounds having, e.g., a dehydroxylated or
- 5 decarboxylated lysine moiety.

Illustrative of polyamines useful as such regulating agents are the following compounds:

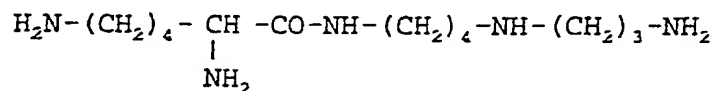
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(weak potassium blocker)

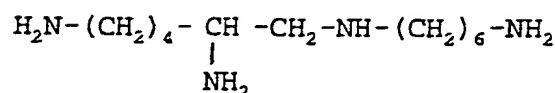
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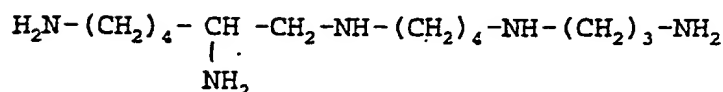
(spermidine-like activity -  
(see Hirsch i.p. experiment)  
but substantially more  
potent)

20 (DD)



(potassium blocker)

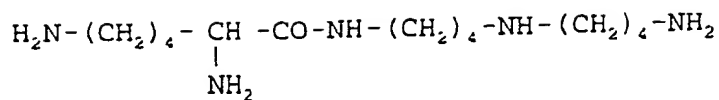
(EE)



25

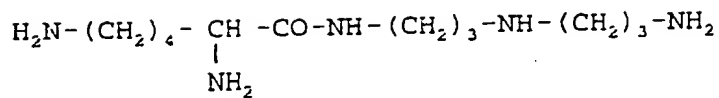
(spermidine-like activity)

(MM)



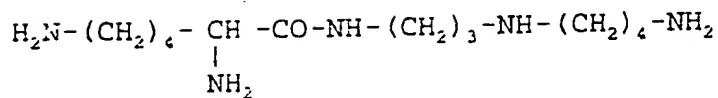
(spermidine-like activity)

5 (NN)



(spermidine-like activity)

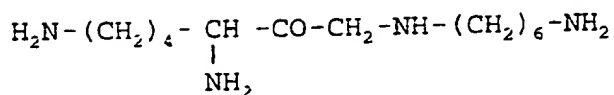
(OO)



(spermidine-like activity)

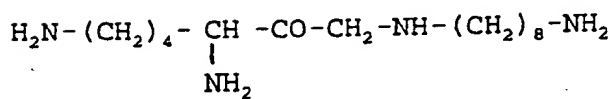
10

(TT)



(potassium channel blocker)

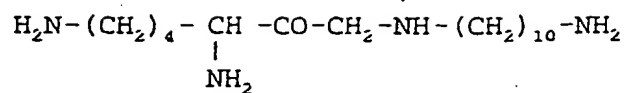
(UU)



(potassium channel blocker)

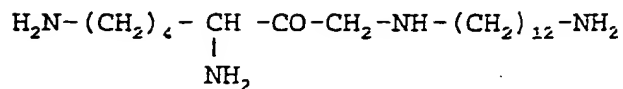
15

(VV)



(potassium channel blocker)

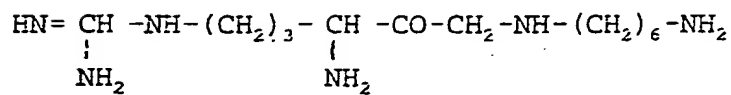
(WW)



(potassium channel blocker)

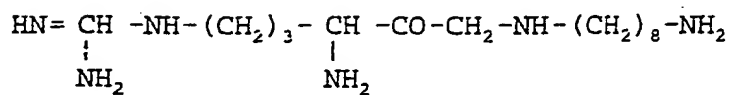
5

(XX)



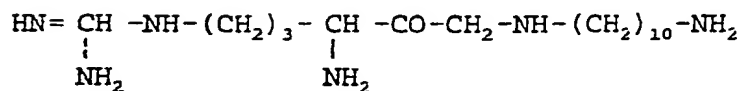
(potassium channel blocker)

(YY)



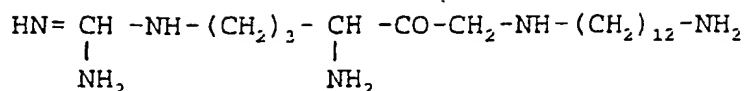
(potassium channel blocker)

10 (ZZ)



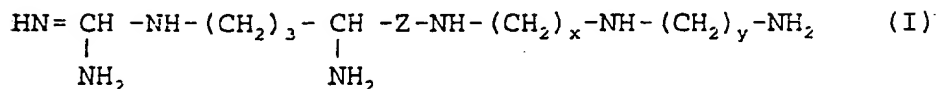
(potassium channel blocker)

(AAA)



(potassium channel blocker)

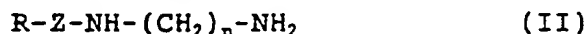
In general, compounds of the formula



- 5 wherein x and y are integers independently selected from 3 or 4 and wherein Z is a carbonyl, CH<sub>2</sub>, or single bond are calcium channel blockers similar to but more potent than spermidine. For example, compound S (which is the most preferred Ca<sup>2+</sup>-blocking compound) was tested as per Example 1B and was found  
10 to block calcium current in squid. In addition, compound S blocked <sup>45</sup>Ca<sup>2+</sup>-uptake in synaptosomes and Ca-dependent transmitter release at the neuromuscular junction synapses. The calcium blocking activity of compound S has been confirmed by single-channel studies. Compounds B and R also demonstrated  
15 such calcium-blocking activity e.g., in Purkinje cells and cerebellar granulocytes, however to different degrees.

In addition, in single-channel studies (eye epithelia), compound R was found to cause inhibition of chloride current, but this is believed to have been secondary  
20 to interference with calcium current (i.e. blockade of the so-called calcium-dependent chloride current was observed).

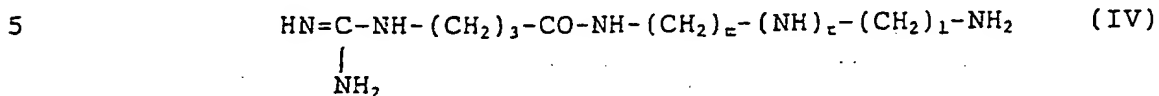
Compounds of the formula



- wherein n is an integer between 6 and 12 inclusive, R is H<sub>2</sub>N-(CH<sub>2</sub>)<sub>4</sub>-CH(NH<sub>2</sub>) or HN=C(NH<sub>2</sub>)-NH-(CH<sub>2</sub>)<sub>3</sub>-CH(NH<sub>2</sub>) and Z is selected  
25 from the group consisting of CO, CH<sub>2</sub>, or a single bond, are potassium current blockers in a manner independent of calcium

ion flow. This was confirmed using each of several compounds within this formula in a purified axon membrane system. The compound wherein  $Z=CH_2$  and  $n=10$  is most preferred.

Compounds of the formula



wherein  $t$  is zero or 1;

10  $l$  is 3 or 4; and

$m$  is 3 or 4 when  $t$  is 1 and  $m$  is zero when  $t$  is zero; were demonstrated to be sodium channel blockers.

Finally, both the calcium blocking compounds and their analogs having a lysine moiety (even though the lysine-based analogs do not block calcium current) have effects observable at a gross level similar to those observed by Hirsch et al., supra, for spermidine but display substantially higher potency than spermidine (e.g. twice as potent). These effects may be related to activity of these compounds on the glutamate (NMDA) receptor, which in turn controls various ion channels.

20 The most preferred lysine-based compounds of Formula II are compounds wherein  $Z=\text{CO}$ ,  $t=1$  and  $m, l=3$  or 4 (independently).

The polyamine compounds of the present invention can be synthesized using well-known and commercially available starting materials and synthetic schemes well-known in the art. Alternatively, polyamine compounds within the scope of the present invention may be obtained from commercial sources.

For example, decarboxylated arginine (agmatine), or arginine ethyl ester, decarboxylated lysine or lysine methyl or ethyl ester can be purchased from Sigma Chemical Co., St. Louis, MO. Other well known polyamines such as spermine, spermidine, 1,6 diaminohehexane and other diaminoalkanes, can also be obtained from Sigma or other commercial sources. Those polyamines which are not themselves active (e.g., spermine) can be used to synthesize active compounds as follows:

One protocol involves using an amine ester in aqueous

solution and reacting it under basic conditions with an appropriate (usually equimolar) amount of a diamine or other polyamine also in aqueous solution (or in liquid form).

See, generally, March, J., Advanced Organic

5 Chemistry, 3d. Ed., 1985, Wiley & Son, New York.

Other synthesis schemes for compounds within the scope of the present invention are disclosed in Eldefrawi, A.T., et al., PNAS, 1988, 85:4910-4913; Hashimoto, Y., et al., Tetrahedron Letters, 1987, 28:3511-3514; and Yamamoto, H., et  
10 al., J. Am. Chem. Soc., 1981, 103:6133-6135 all of which can be employed with only such modifications, if any, as are readily apparent to those of ordinary skill in the art. Additional synthetic schemes may be readily devised based on International Application WO89/07098 published August 10, 1989.

15 Another useful synthetic scheme which can be applied to the present compounds is the one disclosed in Melchiorre, et al., 1989, supra which involves use of a dicarboxylic acid to increase the length of a polyamine (in the presence of  $\text{CH}_3\text{CH}_2\text{OCOC}_1$ , triethylamine and dioxane with addition of HCl and  
20 ethanol, followed by reduction in the presence of boron hydride, dimethyl sulfide and diglyme).

One preferred synthetic scheme for compounds within the scope of this invention that contain a decarboxylated arginine moiety (for example  $\text{H}_2\text{N}-(\text{CH}_2)_3-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{NH}-(\text{CH}_2)_3-\text{NH}-$   
25  $(\text{CH}_2)_4-\text{NH}_2$  or  $\text{H}_2\text{N}-(\text{CH}_2)_3-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$ ) involves reducing the appropriate protected amine ester, e.g.,  $\text{Z}-\text{NH}-(\text{CH}_2)_4-\text{CH}(\text{NH}-\text{Z})-\text{CO}-\text{OEt}$ , (wherein Z is an appropriate protective group such as  $\text{Ph}-\text{CH}_2-\text{O}-\text{CO}-$  wherein Ph is phenyl) in the presence of THF (at -78 degrees C) and di-isobutylaluminum  
30 hydride to the corresponding aldehyde, aminating this product in the presence of sodium cyanoborohydride to produce the desired polyamine which can then be deprotected and purified according to well-known methods.

Alternatively, the protected amino acid (e.g. the  
35 free acid corresponding to the ethyl ester referred to above)

can be first converted to the corresponding mixed anhydride by reacting with, e.g., Et-O-CO-Cl (ethoxycarbonyl chloride) and TEA (triethylamine) and THF (tetrahydrofuran) at -20 degrees C. The anhydride can then be reacted with an alcohol, e.g.,  
5 methanol at -10 degrees C in the presence of sodium borohydride to convert the mixed anhydride to the corresponding alcohol. The alcohol can then be converted to the corresponding halide according to well-known halogenation techniques. The halide can be condensed with the appropriate amine (in the  
10 presence of an acid scavenger such as  $K_2CO_3$  or dimethyl formamide) to yield the target protected compound which can be deprotected by e.g., hydrogenolysis ( $H_2$  palladium/carbon 10%) or where the protecting group is Boc, with trifluoroacetic acid to yield the actual target compound.

15 Compounds within the formula I wherein R does not have an alpha amino group appended to a -CH<sub>2</sub> group and their isomers (e.g.,  $HN=C(NH_2)-NH-(CH_2)_4-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$ ) can be synthesized from the appropriate polyamine (for example,  $H_2N-(CH_2)_4-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$ ) using a guanylation agent such  
20 as 3,5-dimethyl-1-guanyl pyrazole nitrate under ethanol reflux. The target compound can be isolated as the nitrate.  
(Guanylation reaction.)

When used as laboratory reagents the compounds of the present invention can be used in widely ranging units from  
25 nanomolar to no particular upper limit. It will be appreciated that the amount that needs to be used in each instance will depend on the activity of a particular compound (whether the compound is a potent blocker, or modulator), on the sensitivity of the specific channel on which this compound is active  
30 (whether the channel properties are easily modifiable), on the specificity of the activity of the particular compound (whether the compound acts exclusively on one type of channel) and on other factors which are well-recognized in the art to be subject to optimization. Such optimization can be easily  
35 achieved without undue experimentation by well-known methods.



When the compounds of the present invention, including pharmaceutically acceptable salts thereof are used for pharmaceutical purposes (for example to bring about the behavioral modifications or to treat the behavioral disorders referred to herein), they may be incorporated in pharmaceutical compositions in oral, enteral, topical, depot, or parenteral dosage forms containing one or more of the present compounds, in association with one or more pharmaceutically acceptable excipients, fillers, salts, coatings, carriers or diluents such as are customarily used with pharmaceutical preparations, e.g., tablets, sustained-release preparations, gelatin capsules, injectable solutions, suppositories, or coupled to a drug delivery system, etc. The active ingredient dosage ranges will encompass the following preferred ranges: 10-100 mg/kg of a particular compound or compounds in rodents, and 0.05-50 mg/kg in man. It will of course be preferred to use the minimum amount which will accomplish the desired effect in order to avoid as many potential side effects as possible. Moreover, the unit content of each dosage form need not by itself constitute an effective amount of active ingredient since a plurality of dosage forms may be administered to achieve an effective amount in combination. In addition, the amount used and the duration of the treatment will also be subject to optimization and will vary depending on the severity and responsiveness of the condition to be treated, the age, weight, and physical condition of the patient, and often also on the administration route.

The invention is further described below by reference to specific Examples which are intended to illustrate it without limiting its scope:

**EXAMPLE 1: ASSAYS FOR P-CHANNEL BLOCKING ACTIVITY**

**A. Purkinje Cell System**

Adult Hartley guinea pigs (400-600 grams from Camm Research Institute, Wayne, New Jersey) were decapitated with a small animal guillotine under ether or sodium pentobarbital

(Abbott Pharmaceuticals, Inc., N.Chicago, Ill., 40 mg/kg i.p.) anesthesia. A rapid craniotomy was performed to remove the squamous portion of the occipital bone, which allowed the total cerebellar mass, including the cerebellar nuclei, to be detached quickly with a metal spatula. The tissue was then immediately immersed in aerated Krebs-Ringer solution containing 124mM NaCl; 55mM KCl; 1.2mM  $\text{KH}_2\text{PO}_4$ ; 2.4mM  $\text{CaCl}_2$ ; 1.3mM  $\text{MgSO}_4$ ;  $\text{NaHCO}_3$  (26mM); and 10mM glucose. This solution was kept refrigerated at 6° C. The cerebellar mass was then transacted sagittally and a single cell slice about 2mm thick was isolated from the vermis or from one of the hemispheres. The slice was affixed with cyanoacrylate to the bottom of a plexiglass cutting chamber and agar blocks were used to surround the slice, thus providing side support. Once secured, the tissue was immersed in Krebs-Ringer solution at 6° C and further sectioned with an Oxford G501 Vibratome (Ted Pella, Inc., Tustin, CA) to yield about six 200- or 300-micron thick cerebellar slices, containing sagittal sections of all the cerebellar folia in a given rostrocaudal plane as well as central white matter and cerebellar nuclear cells. Following this procedure, the slices were incubated in oxygenated (95%  $\text{O}_2$ ; 5%  $\text{CO}_2$ ) Krebs-Ringer solution at 37° C for about one hour.

After incubation, a slice was transferred to a recording dish such as that described in Llinas, R. et al, J. Physiol., 305:171, 1980. The cerebellar slice was placed in a Sylgard plate (Corning Glass, Corning, New York) at the bottom of the recording chamber and secured with a bipolar stimulating electrode pressing lightly on the white matter. The experiments were conducted at a chamber temperature of 37° C maintained by a surrounding temperature-controlled water bath. The saline (Ringer's) solution used for continuous perfusion was also kept at 37° C.

Various channel blocking agents were used to block conductances under study or to block ionic conductances that would interfere with a particular experiment: Tetrodotoxin

("TTX"  $10^{-6}$ M) was used to block sodium conductance; triethylammonium chloride ("TEA", 30mM), was used to block potassium conductance; nitrendipine ( $10^{-5}$ - $10^{-6}$ M) was used to block the L-channel.

5 Various preparations of compounds to be tested for P-channel blocking activity were introduced in the bath and the flow was turned off for various time periods. These preparations were added in aliquots of 20-40 microliters from a solution containing a particular molar concentration of each  
10 substance to be tested. The volume of the extracellular bath was 4 cc.

Purkinje cells were impaled with recording micropipettes under direct vision using Hoffman modulation microscopy (Hoffman, R., J. Microsc. 110:205-222, 1977). Intracel-  
15 lular recordings were obtained with micropipettes filled with 3M potassium acetate or 1M tetraethylammonium chloride (TEA), and having an average D.C. resistance of 60-80 mega ohms. Synaptic activation of the cells was effected with a bipolar stimulation electrode located on the white matter at the basis  
20 of the folium studied. Direct stimulation of the Purkinje cells was implemented with a high-input impedance ( $10^{12}$  Ohms) bridge amplifier.

In this series of experiments, various concentrations of the compound being tested were used as specified below: The  
25 smaller the amount of active compound that achieves blockage the higher the affinity of the active polyamines for calcium channels. Representative results of the experiments described below are illustrated in Figure 1.

Before the introduction of a polyamine or other  
30 channel blocking compounds in the bath, upon injection of an outward (depolarizing) current pulse, the neurons responded with firing having both sodium-dependent and calcium-dependent spikes in accordance with the normal electrical response of the Purkinje cells in the absence of a P-channel blocker (Fig. 1A).  
35 The cell responses were also measured at several time intervals

after introduction of a polyamine compound in the recording chamber medium. Typically, in the presence of a P-channel blocker, a small depolarizing current (approximately 0.3 or 0.45 nA for 62 msec) generates a burst of potential spikes and a plateau potential, the latter due to the non-inactivating ("persistent") sodium conductance; calcium spikes are substantially reduced or extinguished and there is no calcium-dependent component to the plateau potential (Fig. 1B). Figure 1B was recorded after the addition of a mixture of compounds B and R to a final concentration of 0.8mM.

The apparent absence of the sodium-dependent fast spikes in Fig. 1B that would be expected to follow the first such spikes observed is due to the substantially increased resistance of the cell (indicated by the sodium-dependent plateau potential). Blockage of calcium channels precludes calcium ions from entering the cell and consequently the exit from the cell of potassium ions is not activated. The results in Figure 1B are comparable to addition of the P-channel blocker isolated from funnel-web spider venom, e.g., A. aperta, as disclosed in U.S. Pat. Appln Ser. No. 219,905.

In Figure 1C (upper trace), potassium conductance was blocked with TEA which was also added to the bath (which already contained TTX and a mixture of compounds B and R. The remaining calcium spike is due to the existence of another type of calcium channel in Purkinje cells. (The P-channel has been blocked by a mixture of compounds B and R as in the experiment of Fig. 1B). The spike of Fig. 1C is due to a calcium conductance that is polyamine-resistant (and funnel-web venom resistant) and is therefore not due to the P-channel. This remaining calcium conductance is blocked by the addition of dihydropyridine (Fig. 1C, lower trace) and is also blocked by the addition (to the same preparation, 1D upper trace) of antibiotics such as streptomycin (Fig. 1D, lower trace).

#### B. Electrophysiology with Squid Stellate Ganglia

In squid synapse voltage clamp and current clamp

experiments, the following observations were made:

Action potentials were induced by direct electrical stimulation of the presynaptic nerve bundle of the giant synapse in squid stellate ganglia. The thus evoked postsynaptic action potential is shown in Figure 4 in the absence of any calcium-blocking agents in the bath (trace A). After addition in the 3 cc bath of 0.15 ml of the preparation of a mixture of compounds B and R (described below), the postsynaptic action potential is markedly reduced (trace B) four minutes after such addition and completely extinguished at 6 minutes.

The decrease and eventual extinction of the postsynaptic action potential is attributed to blockage by the P-channel blocking factor (here a mixture of compounds B and R) of the presynaptic calcium channels.

That calcium channels are involved was demonstrated in another type of voltage clamp experiment (Fig. 6) in which the postsynaptic potential in squid synapse (in a preparation containing tetrodotoxin and 3-aminopyridine) is reduced over time after the addition of 0.4 ml of a 1mM solution of the P-channel blocker mixture of compounds B and R in the 3 cc bath. In Figure 6, the squid postsynaptic action potential evoked by a 38.2 mV voltage clamp for 10 msec from a resting potential of -60mV was recorded 1, 6, 10 and 13 minutes after addition of the P-channel blocker. The potential was reduced and eventually extinguished. It should be noted that squid synapse is less accessible to reagents than Purkinje cells; therefore greater amounts of a blocking agent and/or a longer waiting time are necessary for a particular effect to be observed.

Figure 5 shows yet another voltage clamp experiment in which a 28mV/5.5 msec voltage step is applied to the presynaptic terminal (trace C) in a squid synapse preparation, where sodium conductance was blocked with TTX and potassium conductance was blocked with 3-aminopyridine. The applied step voltage generates a compensating inward ionic current in the presynaptic cell (trace B) which is due solely to calcium since

the other conductances are blocked. At the postsynaptic cell a postsynaptic potential (EPSP) is evoked, which is the normal response (upper curve, trace A) in the absence of a P-channel blocker (here a mixture of compounds B and R). At the same time, in the presence of the mixture of compounds B and R, the presynaptic ionic current is also reduced (trace B, upper curve, i.e. curve with the lesser amplitude).

This indicates that the P-channel blocker acts on the presynaptic calcium channels since the time relationship in Fig. 5 of the presynaptic calcium entry to the postsynaptic response remains constant and only the amplitude of the postsynaptic response is reduced in direct relationship to the reduction of the presynaptic calcium current. This means that the P-channel blocker blocks presynaptic calcium channels which impedes calcium influx and consequently the expected transmitter release does not occur. The reduced transmitter release in turn causes a reduced postsynaptic response.

In a current clamp experiment using squid synapse, a fixed amplitude depolarizing current (400 nA, 8.4 msec) is applied to the presynaptic terminal. In the presence of TTX and 3-aminopyridine, the presynaptic potential (calcium spike) is as depicted in Figure 7A, upper trace.

The corresponding postsynaptic potential (EPSP) is depicted in Figure 7B (upper trace). Seven minutes after addition of P-channel blocker (here a mixture of compounds B and R) the presynaptic calcium spike is reduced (7A, lower trace) and so is the EPSP (7B, lower trace). The presynaptic calcium spike after blockage varies in amplitude only. The evoked postsynaptic potential also varies in amplitude. Moreover, the time course of the EPSP after blockage is the same as in the absence of blocker. This indicates that the polyamine P-channel blocking agents act presynaptically and do not have a postsynaptic effect: if a postsynaptic blockade was present, the time course of the EPSP would also be different (in addition to its amplitude being lower).

Figures 2 and 3 demonstrate that the effect of the P-channel blocking agent on the presynaptic calcium current and postsynaptic potential is dose-dependent: both decrease in amplitude with an increasing amount of P-channel blocker used in the extracellular medium. (In Fig. 2 venom was used and in Fig. 3 partially purified *A. aperta* P-channel blocker was used). The partially purified preparation was processed by deproteination from venom (boiling), centrifugation, removal of the pellet, addition to original volume of water followed by twice butanol extraction in 20 volumes of butanol. The aqueous phase was used.

The results described herein are qualitatively the same regardless of the particular P-channel polyamine blocker used. The only difference is the potency of the particular blocker. The mixtures of compounds B and R appeared to be much more potent than spermidine, and, for that reason, these compounds are preferred.

EXAMPLE 2: POLYAMINES USED FOR REGULATION OF CHANNELS

The following compounds were purchased or synthesized and tested for ion channel blocking activity.

The following were obtained from Sigma:

spermidine	$\text{NH}_2-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$
1,6 diaminohehexane	$\text{NH}_2-(\text{CH}_2)_6-\text{NH}_2$
spermine	$\text{NH}_2-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_2$

In addition, L-arginine ethyl ester

$$\begin{array}{c} \text{NH}_2 \\ | \\ \text{HN}=\text{C}-(\text{CH}_2)_3-\text{C}-\text{C}-\text{OCH}_2\text{CH}_3 \\ | \quad | \quad || \\ \text{NH}_2 \quad \text{H} \quad \text{O} \end{array}$$

also from Sigma was used to synthesize compounds B and A, above. Briefly, the synthesis of Compound B involved use of  $3 \times 10^{-3}$  moles of L-arginine ethyl ester which was dissolved in 5ml of 1N NaOH. An equimolar amount of spermidine was added dropwise and the reaction was allowed to proceed at 25°C, pH 14 under stirring. The product was brought to pH 7.4 by addition of 5N HCl. Forty-microliter aliquots of this preparation of

Compound B were used in experiments with the assay system of Example 1.

Compound A was synthesized in the same manner except that an equimolar amount of  $\text{NH}_2-(\text{CH}_2)_6-\text{NH}_2$  was first dissolved in a minimum volume of water before adding to the arginine ester in 1N NaOH.

Compound E (4,3) and its isomer (3,4) can be synthesized by a guanylation reaction, as described above.

In addition, several antibiotics containing polyamine moieties were obtained commercially and tested. These were:

Gentamycin (from Elkins-Sinn, Inc., Cherry Hill, N.J.).

Streptomycin (Pfipharmecs, a division of Pfizer, Inc., New York, N.Y.).

Vancomycin (Eli Lilly Industries, Inc., Carolina, Puerto Rico).

It is anticipated that other antibiotics containing amine groups such as Kanamycin will have the same activities, any differences being of degree rather than kind. Several such antibiotics are commercially available.

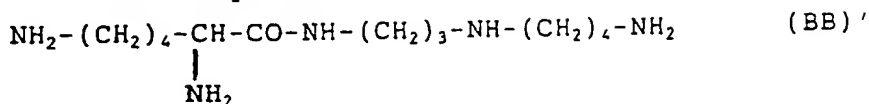
Each of the three antibiotics listed above as having been tested was dissolved in water and 0.1, 0.4, and 0.8 mM amounts were used. 1 mM of vancomycin was used.

Finally, the compounds 4-(3-aminopropyl) morpholine from Aldrich Chemicals Milwaukee, Wisc., and PHTX433 ("PTX433"), a compound described in Eldefrawi et al., 1988, supra (believed to be a glutamate receptor antagonist for locust leg muscle) were also tested.

Amounts of up to 1.0 mM of the morpholine derivative and even larger amounts of PTX433 were used for electrophysiological evaluation of their P-channel blocking activity.

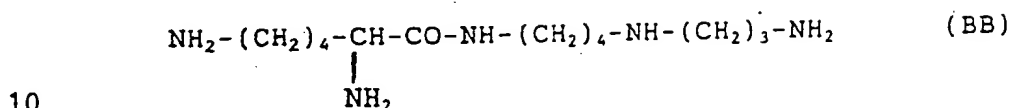


The compounds



5

and



10

were synthesized by reacting an equimolar mixture of lysine and spermidine in water, in the presence of an equimolar amount of a water-soluble carbodiimide namely N-cyclohexyl-N'-((2-morpholinyl)-ethyl)-carbodiimide-methyl-p-toluene sulfonate (Aldrich Chemical Co.) at pH 4.7. The reaction mixture was allowed to remain 20 hours at room temperature under stirring. The product mixture was extracted three times with ethyl acetate (total about 10 volumes) and the aqueous phase was recovered and brought up to pH 7.2. The aqueous phase can be further purified by FPLC (high performance liquid chromatography-Pharmacia) under a 0-1.0 NaCl gradient, pH 7.5 and a flow rate of 1ml/min using a cation-exchange column such as Mono-S (5x50 mm) from Pharmacia. Alternatively, another conventional further purification method can be used. However, the aqueous phase resulting from the above-described final ethyl acetate extraction can be used as is.

Although the foregoing synthesis method does not differentiate between the two compounds (BB) and (BB)' and in all likelihood yields a mixture of these compounds, the activity of the extracted product indicates that both are active. (This mixture and other similar mixtures can be separated and the individual compounds purified by chromatographic techniques using e.g. silica gel or a DOWEX cation exchange column.)

The foregoing technique can be used to synthesize other lysine-based polyamines within the invention.

Another synthetic technique that can be used to yield only compound (BB)' is to first react the lysine with 1,3

diamino propane using, e.g., the above carbodiimide method, and then reacting the product (preferably) after purification with 1,4 diamine butane using one of the methods described above for the arginine-polyamine compounds. The order of use of the  
5 diamines can be reversed for synthesizing the (BB) product. The technique can be applied to synthesis of other compounds of the invention.

EXAMPLE 3: FURTHER STUDIES

Compounds B and R are lethal to rats when  
10 administered at a dose of 50 to 100 microliters. Similar amounts of purified compound BB do not kill these animals. On observation, rats are placid but display no visible movement disorders, and do not extend their limbs when picked up. Compound BB (and/or BB') appeared to have a calming effect  
15 similar to a tranquilizing effect but without much muscle relaxation.

EXAMPLE 4: FLUORESCEIN-LABELLING OF CALCIUM-REGULATING AGENTS

Arginine was labelled by mixing with an equimolar amount of fluorescein in sodium borate buffer, pH 9.5 and  
20 allowing the mixture to react overnight in the dark under stirring. Excess fluorescein was extracted by washing three times with 10 volumes ethyl acetate per wash. The thus labelled arginine can be used to synthesize compounds of Formula I. Lysine ethyl ester can be similarly labelled.  
25 Thus, labelled Formula I compounds can be used in fluorescence experiments, using methods well-known in the art.

EXAMPLE 5: RESULTS OF FURTHER ELECTROPHYSIOLOGICAL STUDIES

P-channel activity was recorded in the system of Example 1 in the absence and presence of various amounts of the  
30 compounds listed in Example 2.

The results for the P-channel were as follows:

Spermine and 1,6 diaminohehexane did not block the P-channel at the concentrations tested (up to about 1 mM). The calcium conductances attributable to P-channels were the same  
35 in the presence and in the absence of these compounds. By

contrast, spermidine blocked the P-channel selectively but not with high potency (compared to the potency of spider venom or purified P-channel blockers from venom).

4-(3-aminopropyl)morpholine did not block the P-channel up to a concentration of 1 mM. PHTX433 did not block the P-channel up to a concentration of 10 mM in Purkinje cells, but blocked the postsynaptic response in squid (about 300 micromolar).

By contrast, the mixture of compounds B and R was a potent P-channel blocker producing complete P-channel blockage at concentrations lower than 0.5 mM. The other channels (sodium, potassium) were not affected. The minimum effective concentrations of the P-channel blocking agents of this invention are micromolar or less; this can be determined by routine experimentation using serial dilutions of these agents. Preferred are concentrations up to 1mM but it should be appreciated that the choice of concentration of each agent is subject to optimization as is well-known in the art and also depends on whether partial or total P-channel blockage is desired.

The antibiotics block the sodium channel at concentrations below 1 mM. None of the antibiotics tested block the P-channel at concentrations below 1 mM although P-channel blockage is seen (as well as L- and sodium channel blockage) when the concentration of the aliquot introduced in the bath is higher than 10 mM. In no instance was P-channel blockage by the antibiotics potent or specific, despite the presence of the arginine-like moiety  $\text{HN}=\text{C}(\text{NH}_2)-\text{NH}-$  in streptomycin and of various amine groups in the other antibiotics.

#### 30 EXAMPLE 6:

A mixture of compounds (BB) and (BB)' was administered to a rat in amounts ranging between 50 and 100 mg/kg. The rat was subsequently injected with 40mg/kg (actual volume: 0.2 ml) of sodium phenobarbital (NEMBUTAL), a dose which normally produces total anesthesia, and in many cases

death. The (BB)/(BB)'-injected rat by contrast, did not become unconscious upon injection. It took two additional doses of nembutal to anesthetize this rat. The experiment was repeated except this time two rats were injected with a mixture of compounds (BB) and (BB)', one with the same dose as was administered in the previous experiment and one with twice that dose. Immediately after injection, the first rat appeared normal and the second exhibited reduced activity on observation. After each was injected with 40mg/kg of nembutal, the first rat showed no effect but the second appeared to wake up. Subsequent 40mg/kg injections of Nembutal caused the first rat to become anesthetized at a total dose of 160mg/kg but the second rat received a total of 200 mg/kg before he was anesthetized, and received 240 mg/kg before he was killed. The normal LD-50 for Nembutal in rats is about 60 mg/kg. Therefore this dose is enormous by comparison. The implication is that mixtures of compounds (BB) and (BB)' cause resistance to barbiturate action. Furthermore, the mixture causes animals to be serene without being tranquilized, in that it does not cause much muscle relaxation and has potential utility as a prototype drug for anxiolysis.

EXAMPLE 7: <sup>45</sup>Ca BLOCK OF SYNAPTOSOMES

A. Preparation of Synaptosomes

Cerebral cortex from three rats was homogenized in 0.32 M sucrose using a manual driven Teflon glass homogenizer. The homogenate was centrifuged at 4° C, 3000g for 10 minutes, and the supernatant was further centrifuged at 50,000g for 20 minutes. The pellet was resuspended in sucrose 0.32M and seeded in a 0.8-1.4 M sucrose gradient. After 2 hours of centrifugation at 25,000g, the bands in 0.8-1; 1-1.2 and 1.2-1.4 M layers were pooled and slowly diluted, 1:4 in NaCl 132mM  $\text{Ca}^{2+}$  free solution. After 35,000g centrifugation for 15 minutes, the pellet was resuspended in (A) Choline Cl 132 mM,  $\text{CaCl}_2$  1.2 mM or in Choline Cl 65mM  $\text{CaCl}_2$  1.2 mM solution. Samples from A and B suspension (500 ul, 1mg/ml of protein)

were incubated for 10 minutes at 32°C with or without the tested toxin.

B.  $^{45}\text{Ca}^{2+}$  Uptake

In nerve terminals, calcium influx into synaptosomes can be estimated by measuring the  $^{45}\text{Ca}^{2+}$  uptake when synaptosomes are depolarized by K.

$^{45}\text{Ca}^{2+}$  uptake was started by the addition of 0.3uCi/uMol of  $^{45}\text{Ca}^{2+}$  diluted in 200 ul Na ringer for basal uptake or Na K ringer for K stimulated uptake (final concentration 65mM). The  $\text{Ca}^{2+}$  uptake was stopped after 15 or 30 seconds with the addition of 3 ml of EGTA (ethylene glycol bis (amino-ethyl-ether) N, N, N', N'-tetra acetic acid) (4mM) buffer solution. This suspension was rapidly filtered under vacuum through Whatmann GF/B filter paper and washed 3 times with the washing solution (Choline Cl 132 mM; KCl 5mM,  $\text{CaCl}_2$  1.2mM,  $\text{MgCl}_2$  1.3mM, glucose 10mM, Tris 5mM pH 7.4). Filters were dried and immersed in scintillation fluid and counted in a scintillation counter. Compound S was prepared by processes similar to those described in Nakanishi et al., International Patent Application No. WO89/07098, particularly pages 18-19 and Yamamoto et al., J. Am. Chem. Soc., 1981, 103:6133-6136, starting with  $\text{H}_2\text{N}-(\text{CH}_2)_3-\text{NH}_2$ . For comparison, FTX was purified chromatographically from the crude venom of American funnel-web spiders (Spider Pharm, Black Canyon, AZ).

C. K Stimulated  $^{45}\text{Ca}^{2+}$  Uptake by Synaptosomes

1. Compound S

Compound S was an effective inhibitor of the K induced  $^{45}\text{Ca}^{2+}$  uptake in cerebral cortex synaptosomes. Submillimolar concentrations of Compound S were capable of blocking the  $^{45}\text{Ca}^{2+}$  uptake in a dose dependent manner.

2. Comparative Data

FTX (0.7 ul/ml) reduced  $^{45}\text{Ca}^{2+}$  uptake to the same level as the non-depolarized synaptosomes. However, 100uM Cd was more effective since it also blocked part of the non K dependent uptake.

As an additional control, the effect of spermidine was studied. A concentration up to 3mM of spermidine lacked of any effect on neuromuscular transmission. Spermidine showed no significant blocking effect.

5 This demonstrates that cerebral cortex synaptosome  $\text{Ca}^{2+}$  is sensitive to Compound S in a manner similar to FTX. However, Compound S can be readily synthesized and is identifiable, unlike FTX.

10 EXAMPLE 8: EFFECT ON  $\text{Ca}$  DEPENDENT TRANSMITTER  
RELEASE IN THE NEUROMUSCULAR JUNCTION

A. Muscle Preparation

Nerve-muscle preparations were obtained from male Swiss mice (25-30g) by removal after cervical dislocation and pinning out in a Sylgard (R) coated chamber containing 1-2 ml  
15 of physiological solution consisting of (millimolar) NaCl, 135; KCl, 5;  $\text{CaCl}_2$ , 2;  $\text{Mg}_2\text{SO}_4$ , 1;  $\text{NaHCO}_3$ , 12;  $\text{NaH}_2\text{PO}_4$ , 1; d-glucose, 11; pH 7.4 kept at room temperature. The solution was oxygenated by continuous bubbling with a mixture of 5%  $\text{CO}_2$  and 95%  $\text{O}_2$ . Intracellular recordings of miniature endplate  
20 potentials and evoked endplate potentials were performed. Glass microelectrodes filled with 2M KCl of 5 to 15 Mohm resistance were used. The electrodes were inserted into the muscle fibers near the endplate regions, which were located visually by the ends of intramuscular branches of the phrenic  
25 nerve. The mean quantal content (m) of transmitter release was measured in muscles incubated in low  $\text{Ca}^{2+}$  (1.2 mM) high  $\text{Mg}^{2+}$  (6 mM) based on the failure method, where  $m = \log e N/N_f$  and N is the total number of nerve stimuli and  $N_f$  is the total number of endplate potentials failure as described in Martin, R.R.,  
30 Physiol. Rev., 1966, 46:51-66. The phrenic nerve was stimulated at 0.5 Hz. Miniature endplate amplitudes were corrected assuming a -80 mV membrane potential and their frequency of appearance counted over 0.5 to 3 minutes. The Levator auris muscle, described in Angaut-Petit et al., Neuro.  
35 Sci. Lett., 1987, 82:83-88, was used to study the presynaptic.

motor nerve terminals currents. The presynaptic currents were recorded with glass microelectrodes filled with 2M NaCl of 5 to 15 Mohm resistance placed inside the perineural sheath of small nerve branches near the endplate areas described by Mallori, J. Physiol. (London), 1985, 363:565-575; and Penner et al., Pflugers Arch, 1986, 806:190-193. The nerves were stimulated using suction electrodes coupled to a pulse generator with associated stimulus isolation unit (0.1 msec). The recording microelectrodes were connected to an Axoclamp 1B amplifier. The signals were digitized (Tecmar labmaster A/D converter), stored and analyzed by a computer.

## B. Electrophysiology

### 1. Presynaptic Currents

Nerve muscle preparations were incubated in normal ringer plus curare, 30  $\mu$ M, tetraethyl ammonium 10mM, 3-4, diaminopyridine 250  $\mu$ M, and procaine 100  $\mu$ M. Presynaptic current was recorded (Figure 8, trace a). 300  $\mu$ M of compound S was added to the solution, and presynaptic current was measured (Figure 8, trace b).

Calcium content was increased to 6 mM in the presence of Compound S, and presynaptic current was measured (Figure 8, trace c).

Submillimolar concentrations of compound S were also effective in blocking the slow long lasting  $\text{Ca}^{2+}$  component of the presynaptic currents. The blocking effect of the toxin was overcome by increasing the calcium concentration in the bath solution in a manner similar to that for other divalent cations as reported by Lin et al., Proc. Nat'l Acad. Sci. USA, 1990, 87:4538-4542.

### 2. Transmitter Release

#### a. Compound S

Synthetic arginine-polyamine (spermidine) adducts were found to have FTX-like activity. Quantal content of transmitter release was inhibited in a dose dependent manner with low (millimolar) concentrations of Compound S.

#### b. Comparative Data

The inhibitory action of FTX on synaptic transmission was further characterized by studying the effect of different concentrations of FTX on quantal content of evoked release.

- 5 The muscles were incubated in a low  $\text{Ca}^{2+}$ /highMg solution. The control m values were between 2.1 and 3.8. A 50% inhibition of the quantal content of evoked release was obtained with submicromolar concentration of FTX.

- 10 In contrast, evoked neuromuscular transmission was completely insensitive to omega-conotoxin (5  $\mu\text{M}$ ) tested under similar conditions as described by Protti et al., Brain Res., 1991, 557:336-339.

#### 3. Spontaneous Transmitter Release

##### a. Compound S

- 15 Spontaneous transmitter release was also affected by Compound S. A 50 to 100% increase in miniature frequency was observed in muscles treated with 0.5  $\mu\text{l/ml}$  of purified toxin or with 1 mM of Compound S. The amplitude of the miniature endplate potentials was substantially reduced (over 40%)  
20 without major alterations in their time course.

- Compound S exhibits high potency in blocking synaptic transmission and presynaptic  $\text{Ca}^{2+}$  currents at the mouse neuromuscular junction, as does FTX. However, Compound S can be readily synthesized and characterized and is therefore  
25 readily available.

#### 4. Further Comparative Data

- Hemidiaphragms incubated in normal Ringer were treated with 1  $\mu\text{l/ml}$  of FTX. After a few minutes muscle contraction elicited by nerve stimulation was abolished.  
30 Intracellular recording showed that nerve stimulation failed to trigger transmitter release in a high percentage of trials. When transmitter release was evoked, small endplate potentials were recorded with fluctuating amplitude multiples of the miniature endplate potential amplitude. Neuromuscular  
35 transmission and contraction was restored a few minutes after



washing the preparation with normal solution.

EXAMPLE 9: POTASSIUM CHANNEL ACTIVITY BASED ON PURIFIED

AXON MEMBRANE

A. Membrane Fractionation

5 Myelin, periaxolemmal and axolemmal enriched fractions were purified from rat lower brain stem dissected free of visible grey matter. These membrane fractions were all isolated from the same preparation by a modification of the procedure of Detskey et al., Neurochem. Res., 1988, 13:449-454,  
10 originally formulated for the isolation of crude axolemmal enriched fractions. The modifications include the use of HEPES instead of TES buffer, the initial centrifugation step was in 0.9M sucrose (BRL, Gaithersburg, MD) rather than 1.0M and that after the first centrifugation step the floated membranes were  
15 rehomogenized in 0.85M sucrose and recentrifuged. The resultant floating membrane pads were washed in 10mM HEPES pH 7.4 containing 5mM EDTA. The membranes were then osmotically shocked in this buffer for 1.5 hours. The overnight discontinuous density gradient centrifugation step described in  
20 the original procedure was carried out on a gradient of 15, 24, 28, 32 and 37% sucrose. The sucrose concentrations were defined by density determined using a refractometer (Fisher Scientific, Pittsburgh, PA).

The myelin, sedimenting on the 15-24% sucrose  
25 interface, and crude axolemma, sedimenting at the 28-32% sucrose interface, isolated by this procedure were purified further. The myelin was osmotically shocked with distilled water and sedimented twice at 12,000Xg in a Beckman Instruments (Wakefield, MA) Model J2-21 centrifuge to remove residual  
30 microsomal contaminants and layered over 0.75M sucrose. The material was centrifuged in a SW-28 rotor at 75,000 xg for 1.5 hours in a Beckman L7-55 ultracentrifuge. The band floating on 0.75M sucrose was taken as purified myelin.

The axolemmal subfractions were purified from crude  
35 axolemma after its lysis for 30 minutes in 5mM Tris-HCl, pH

8.3, containing 0.1mM EDTA. The lysate was made isotonic by the addition of 1/4 volume of 40% sucrose and was layered over 0.65M, 0.8M and 1.0M sucrose and centrifuged at 75,000 Xg for 1.5 hours. Since most myelin fractions have densities less than 0.65M this step was included to preclude residual myelin contaminants, although usually little material was observed on this layer. The material on the 0.8M and 1.0 M sucrose layers were defined as the 0.8M and 1.0M subfractions. These membranes were diluted in deionized water and centrifuged at 15,000 xg for 20 minutes to remove any small membrane contaminants. The extrinsic membrane proteins of these fractions were identified by treatment of the membranes with Na<sub>2</sub>CO<sub>3</sub>, as described in Sapirstein et al., J. Neurochem., 1988, 51(3):925-933, and the extracted proteins precipitated with 10% cold trichloroacetic acid..

#### B. Electrophysiology

The initial homogenate contained 5mM  $\beta$  mercapto-ethanol to retain active ion channels. Electrophysiologic analysis of ion channel activity was carried out by the fusion of membrane subfractions. Membranes were resuspended into 400mM sucrose using a polytron for 15 seconds to form osmotically-loaded vesicles for fusion to the bilayer with lipid bilayers coating the surface of glass pipettes, as described by Llinas et al., Proc. Nat'l Acad. Sci. USA, 1989, 86:1689-1693. The final concentration of the membrane subfractions on the cis side of the bilayer was approximately 13ug/ml. Micropipettes with a tip diameter of approximately 1 micron (Corning 7052 glass, Corning, NY) were pulled using a Kopf Model 720 Electrode Puller (Tujunga, CA). Lipid bilayers were formed using a 1:1 mixture of phosphatidyl ethanolamine and phosphatidyl serine (Sigma Chem. Co., St. Louis, MO) using the "tip-dip" technique of Coronado and Latorre as described in Biophysical Jour., 1983, 43:231-236. Voltages were applied via an Axon Instruments (Foster City, CA) A12120 patch-clamp amplifier with a 100GOhm head stage. The bathing solution was held at ground.

The data obtained in the channel studies were amplified to 100 Mv/pA and the membrane current was recorded on a Neurocorder DR-384 (NeuroData, New York) for subsequent analysis. The data were filtered at 1kHz and digitized at a rate of 25,000 samples/sec. The digitized records were then analyzed by computer using the pClamp program (Axon Instruments). Experiments were performed using symmetrical bathing solutions of 150mM of KCl (for K<sup>+</sup> conductances). Channel conductances are determined as pico-siemans (pS), calculated as  $pS = pA/V$ .

Results are illustrated in Figures 9A, B, C, and D. Axolemma membrane vesicles were added to the outside bathing solution and were allowed to fuse with the lipid bilayer. Channel-like activity was measured at different holding potentials. After determining a control value (Figure 9A), compound (ZZ) at a concentration of 1mM was added to the bathing solution. Channel-like activity was monitored by the application of 50mV/ msec at one (Figure 9B) and three (Figure 9C) minute intervals after the addition of compound (ZZ). Channel-like activity was monitored by the application of 75 mV/ msec at a three-minute interval (Figure 9D) after the addition of compound (ZZ).

At this concentration, compound (ZZ) was found to abolish potassium channel activity in this membrane within three minutes.

EXAMPLE 10: SODIUM CHANNEL ACTIVITY BASED ON PURIFIED AXON MEMBRANE

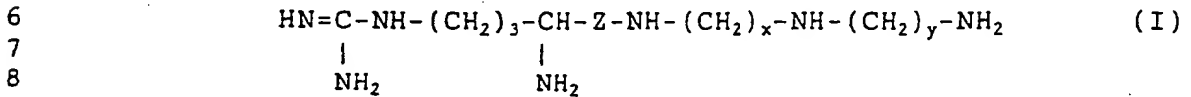
The procedure of Example 9 is followed. However, Na<sup>+</sup> channel analysis is performed. Na<sup>+</sup> channel analyses are performed by extending channel open times by the addition of batrachotoxin (BTA) (Laboratory of Bioorganic Chemistry, NIDDK, Bethesda, MD) at the final concentration of 120nM, along with the membrane vesicles, to the *cis* side of the bilayer as described by O'Leary et al., Mol. Pharmacology, 1989, 36:789-795.

After determining a control value (Figure 10), compound (ZZ) at a concentration of 1mM is added to the bathing

solution. Channel-like activity is monitored, and Na<sup>+</sup> channel activity in this membrane is abolished.

WHAT IS CLAIMED IS:

1                   1.    A method for regulating calcium transport across  
2    cellular membranes possessing calcium channels comprising  
3    exposing a cell membrane possessing said channels to the  
4    presence of at least one non-aromatic polyamine compound of the  
5    formula:



9    wherein x and y are integers independently selected from 3 or 4  
10   and wherein Z is a carbonyl, CH<sub>2</sub>, or single bond; said poly-  
11   amine being present in an amount effective to block conductance  
12   of said channel without having a substantial direct effect on  
13   the conductance of other ions.

1                   2.    The method of claim 1 wherein said calcium  
2    channels are P-channels.

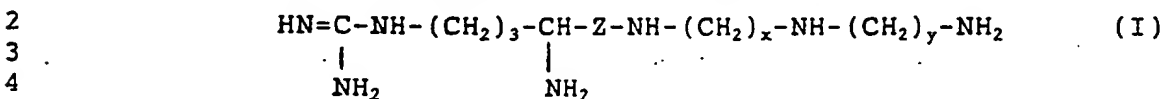
1                   3.    The method of claim 1 wherein both x and y are  
2    3.

1                   4.    The method of claim 1 wherein x is 4 and y is 3.

1                   5.    The method of claim 1 wherein said polyamine is  
2    a mixture of two polyamines according to said formula, the  
3    first polyamine having x=4 and y=3 and the second polyamine  
4    having x=3 and y=4.

1                   6.    The method of claim 1 wherein x, y=3 and Z=CO.

1                   7.    A compound of the formula:



5    wherein x, y are integers independently selected from 3 or 4  
6    and wherein Z is carbonyl, CH<sub>2</sub>, or a single bond.

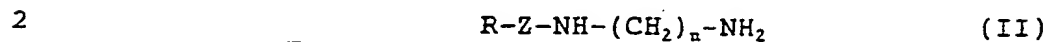
1                   8.    The compound of claim 7 wherein x, y=3 and Z is  
2    carbonyl.

1                   9.    The compound of claim 7 wherein x, y=3 and Z is  
2    a single bond.

1                   10.   The compound of claim 7 wherein x=3, y=4 and  
2    Z=CO.

1                   11.   The compound of claim 7 wherein x=4, y=3 and  
2    Z=CO.

1                   12.   A compound of the formula:



3    wherein n is an integer between 6 and 12 inclusive, R is H<sub>2</sub>N-  
4    (CH<sub>2</sub>)<sub>4</sub>-CH(NH<sub>2</sub>) or HN=C(NH<sub>2</sub>)-NH-(CH<sub>2</sub>)<sub>3</sub>-CH(NH<sub>2</sub>) and Z is selected  
5    from the group consisting of CO, CH<sub>2</sub>, or a single bond.

1                   13.   The compound of claim 12 wherein Z=CH<sub>2</sub> and n=6.

1                   14.   The compound of claim 12 wherein Z=CH<sub>2</sub> and n=8.

1                   15.   The compound of claim 12 wherein R=H<sub>2</sub>N-(CH<sub>2</sub>)<sub>4</sub>-  
2    CH(NH<sub>2</sub>), Z=CH<sub>2</sub> and n=10.

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16. The compound of claim 12 wherein  $Z=CH_2$  and  $n=12$ .

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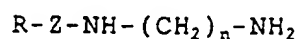
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17. A method for regulating potassium transport across cellular membranes possessing potassium channels comprising exposing a membrane possessing said channels to the presence of at least one compound of the formula:



wherein  $n$  is an integer between 6 and 12 inclusive,  $R$  is  $H_2N-(CH_2)_4-CH(NH_2)$  or  $HN=C(NH_2)-NH-(CH_2)_3-CH(NH_2)$  and  $Z$  is selected from the group consisting of  $CO$ ,  $CH_2$ , or a single bond;

said compound being present in an amount effective to block conductance of said channels.

1

18. The method of claim 17 wherein  $Z=CH_2$ .

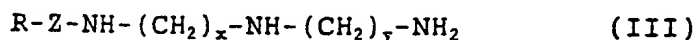
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19. The method of claim 18 wherein  $Z=CH_2$  and  $n=10$ .

1

20. A compound of the formula:

2



3

wherein  $R$  is  $H_2N-(CH_2)_4-CH(NH_2)$  or  $HN=C(NH_2)-NH-(CH_2)_3-CH(NH_2)$ ;

4

$Z$  is carbonyl,  $CH_2$  or a single bond; and

5

$x, y$  are integers independently selected from 3

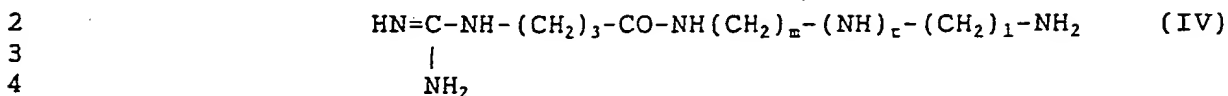
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or 4.

1 21. The compound of claim 20 wherein R is

2  $\text{H}_2\text{N}-(\text{CH}_2)_4-\text{CH}(\text{NH})$ .

1 22. A compound of the formula:



5 wherein t is zero or 1;

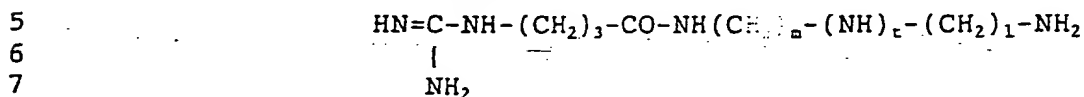
6 l is 3 or 4; and

7 m is 3 or 4 when t is 1 and m is zero when t is zero.

1 23. The compound of claim 22 wherein t and m are  
 2 zero.

1 24. The compound of claim 22 wherein t is 1 and n, m  
 2 are 3.

1 25. A method for regulating sodium conductance in  
 2 cell membranes possessing sodium channels comprising exposing a  
 3 cell membrane possessing said channels to the presence of a  
 4 compound of the formula:



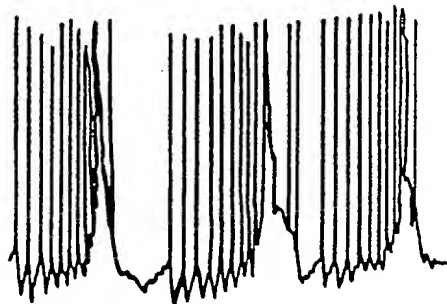
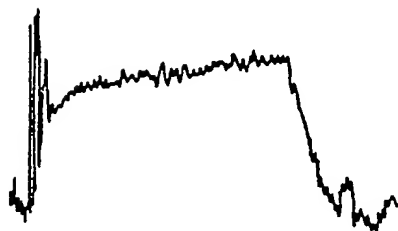
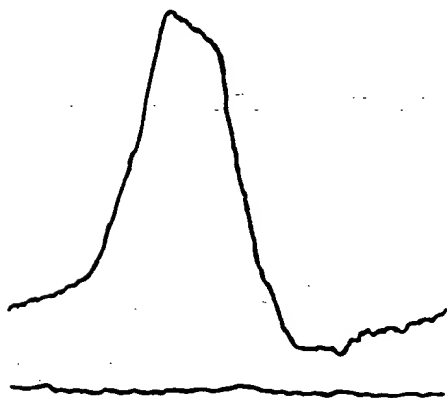
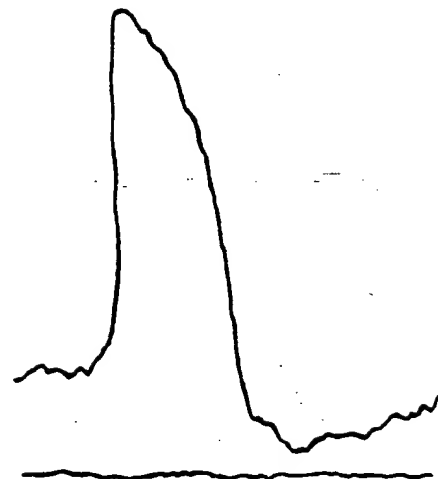
8 wherein t is zero or 1;

9 l is 3 or 4; and

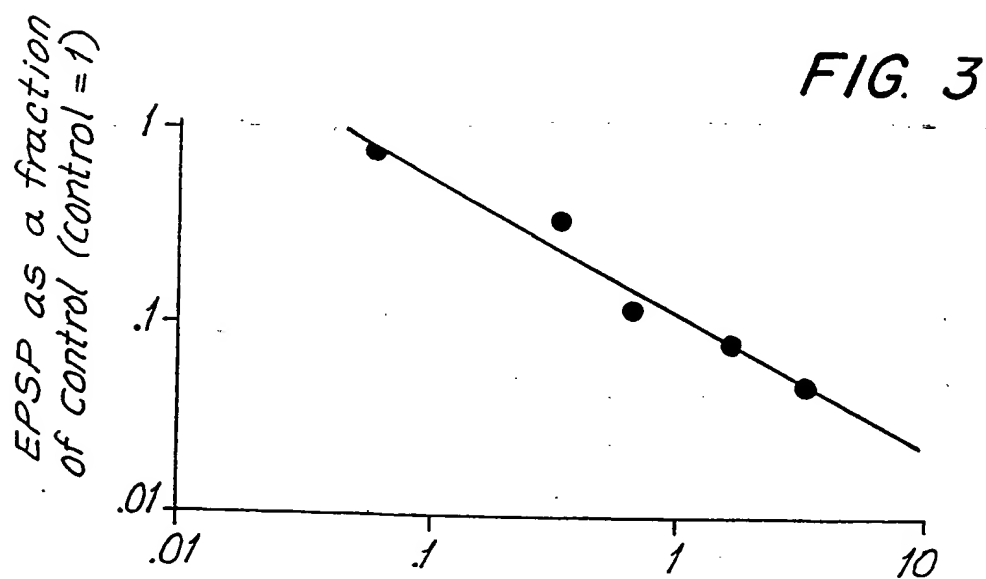
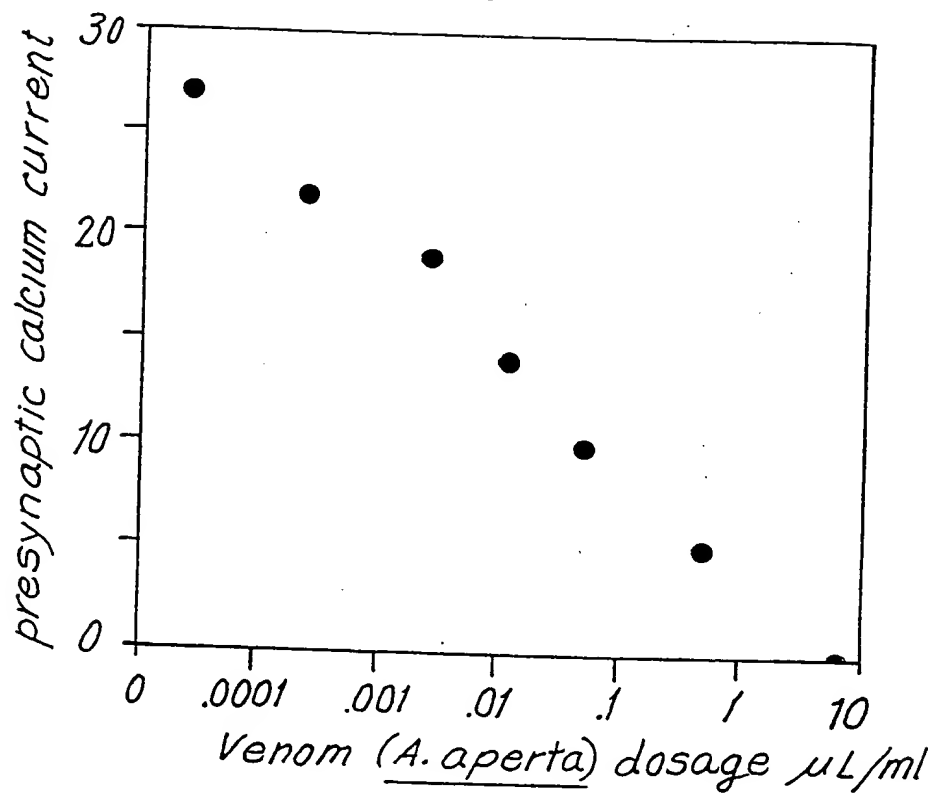
10 m is 3 or 4 when t is 1 and m is zero when t is zero;

11 said compound being present in an amount suffi-  
 12 cient to block conductance of said channels.



*FIG. 1A**FIG. 1B**FIG. 1C**FIG. 1D*

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purified P-channel blocking agent  
from *A. aperta* venom

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FIG. 4

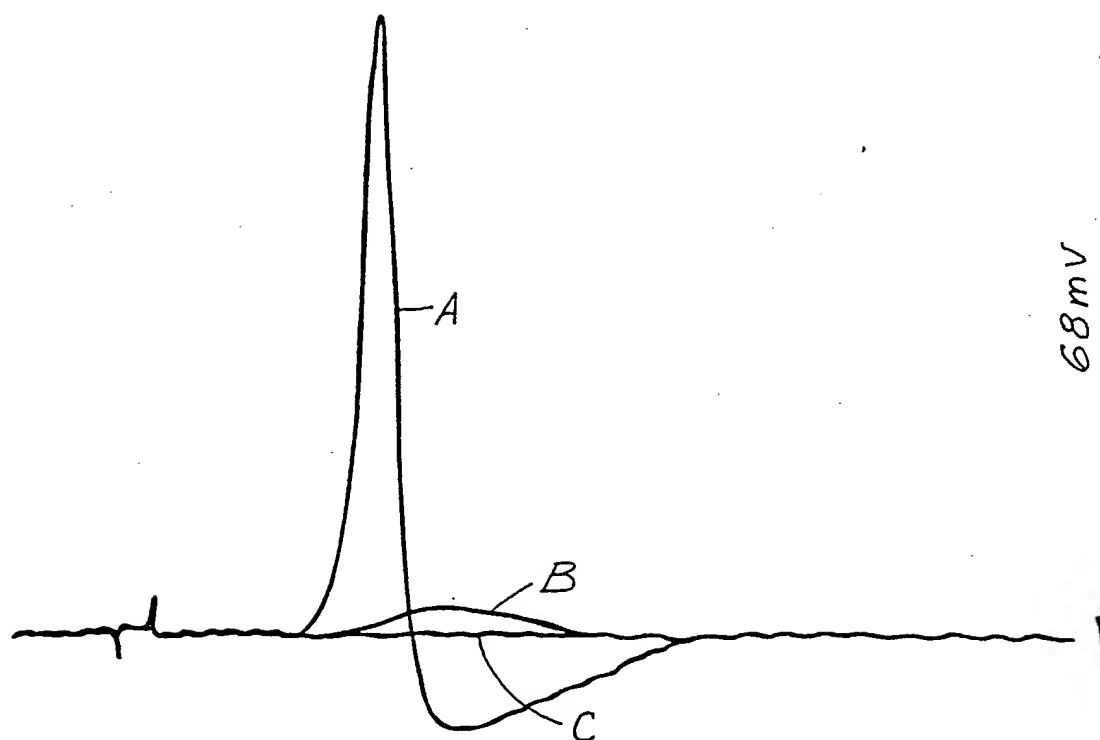
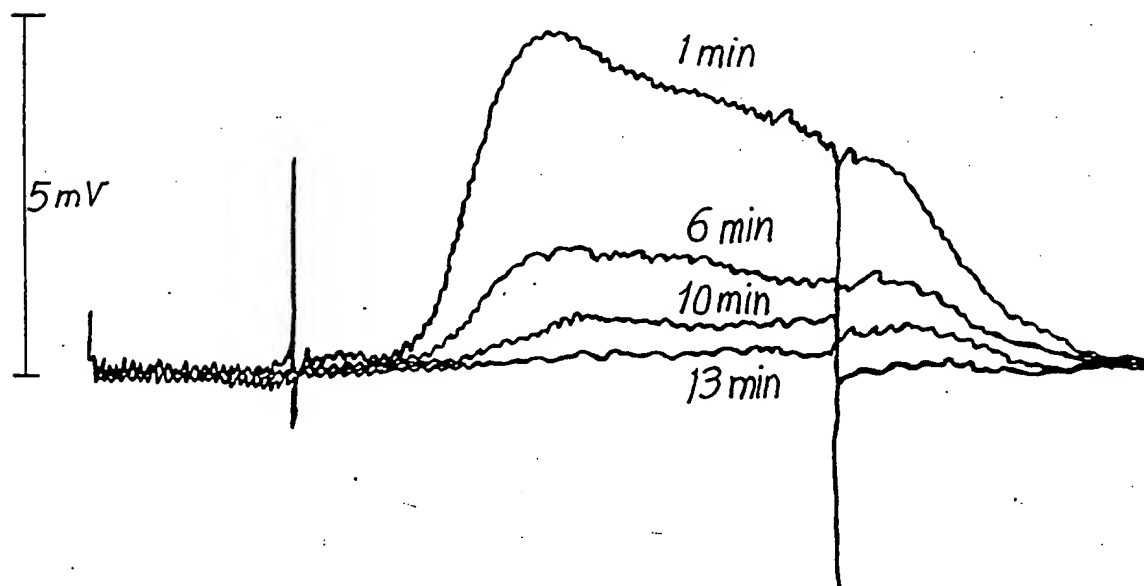


FIG. 6



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FIG. 5

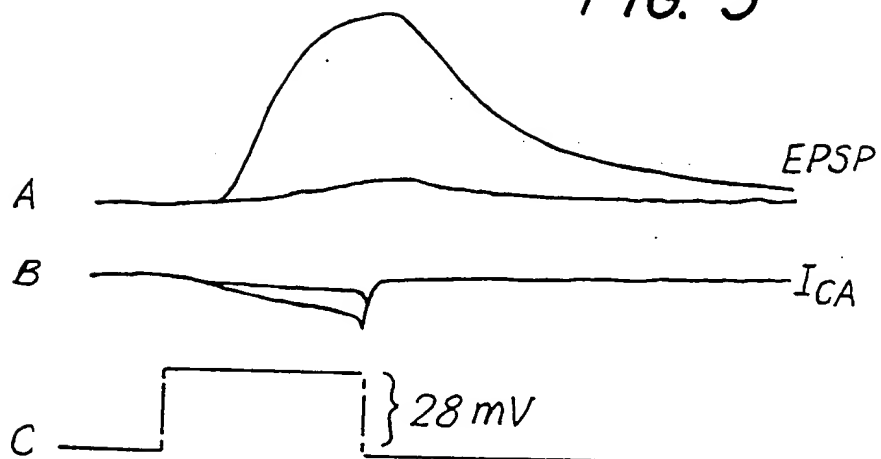
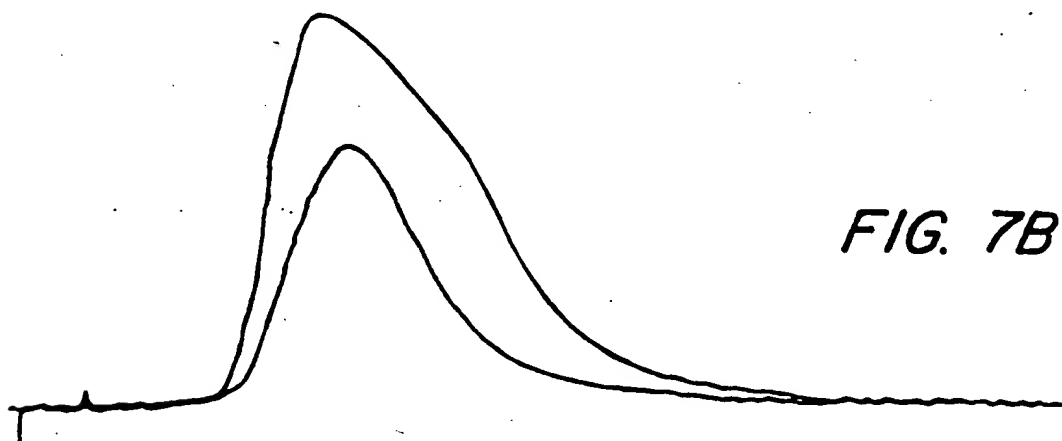


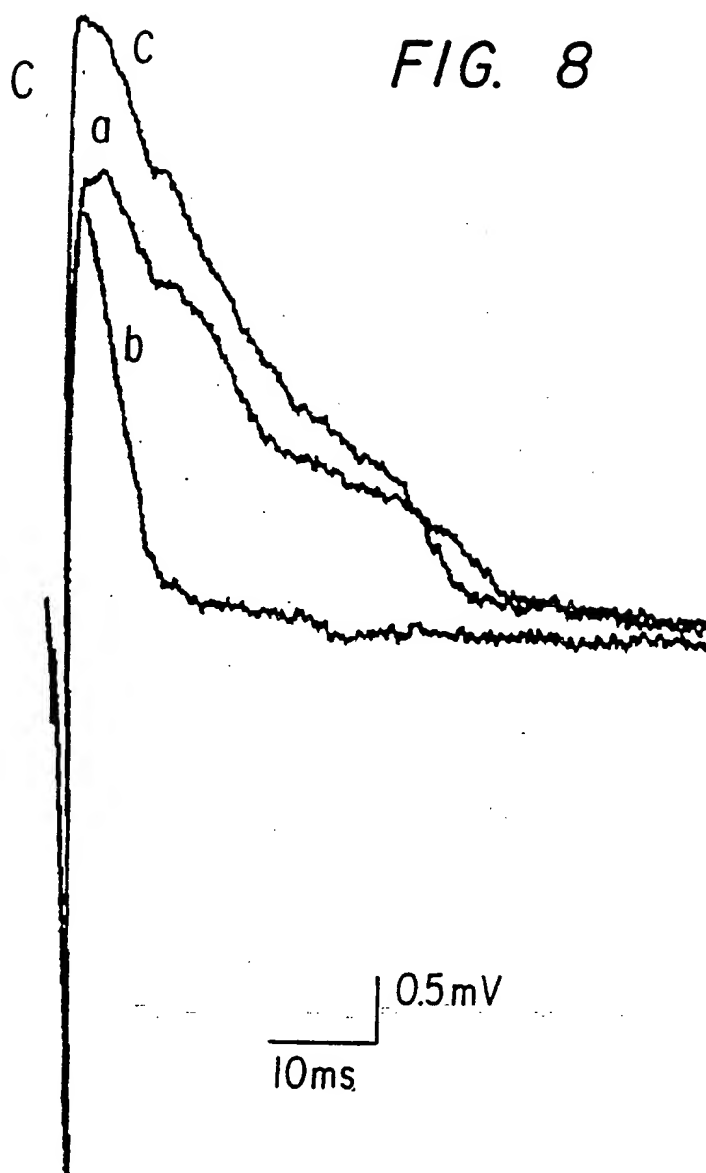
FIG. 7A



FIG. 7B



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FIG. 9A



PURIFIED AXOLEMMA MEMBRANE

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FIG. 9B

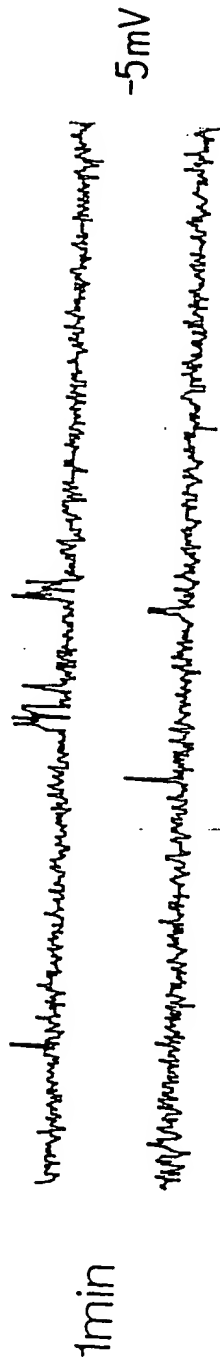
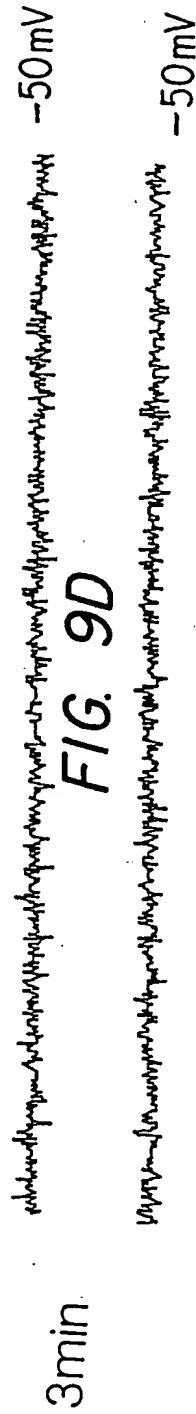
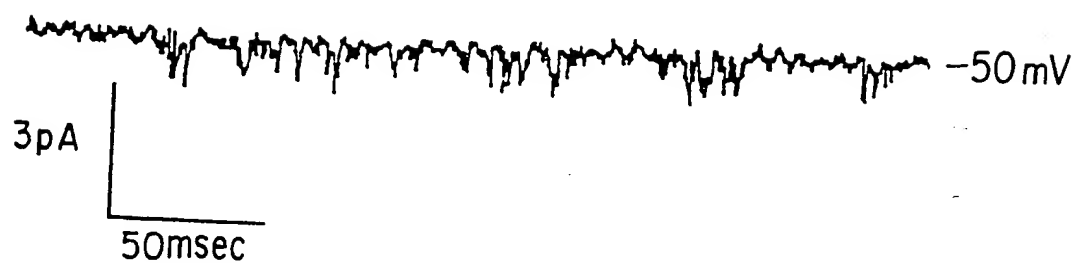


FIG. 9C



50ms/div.

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*FIG. 10*

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US90/11352

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 31/16, 31/155; C07C 237/06, 257/14

US CL : 514/626, 634, 674; 564/197, 240, 512

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/626, 634, 674; 564/197, 240, 512

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS Online - structural formulas

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 3,574,747 (DENNING) 13 April 1971. See abstract.	1-25

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Further documents are listed in the continuation of Box C.

☐

See patent family annex.

\* Special categories of cited documents:

\*A\* document defining the general state of the art which is not considered to be part of particular relevance

\*E\* earlier document published on or after the international filing date

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\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

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\*X\*

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\*&amp;\*

document member of the same patent family

Date of the actual completion of the international search

23 MARCH 1993

Date of mailing of the international search report

19 APR 1993

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